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Prevention of liver ischemia reperfusion injury by a combined thyroid hormone and fish oil protocol☆

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Abstract

Several preconditioning strategies are used to prevent ischemia–reperfusion (IR) liver injury, a deleterious condition associated with tissue resection, transplantation or trauma. Although thyroid hormone (T_3) administration exerts significant protection against liver IR injury in the rat, its clinical application is controversial due to possible adverse effects. Considering that prevention of liver IR injury has also been achieved by n-3 polyunsaturated fatty acid (n-3 PUFA) supplementation to rats, we studied the effect of n-3 PUFA dietary supplementation plus a lower dose of T_3 against IR injury. Male Sprague-Dawley rats receiving fish oil (300 mg/kg) for 3 days followed by a single intraperitoneal dose of 0.05 mg T_3/kg were subjected to 1 h of ischemia followed by 20 h of reperfusion. Parameters of liver injury (serum transaminases, histology) and oxidative stress (liver contents of GSH and oxidized proteins) were correlated with fatty acid composition, NF-κB activity, and tumor necrosis factor-α (TNF-α) and haptoglobin expression. IR significantly modified liver histology; enhanced serum transaminases, TNF-α response or liver oxidative stress; and decreased liver NF-κB activity and haptoglobin expression. Although IR injury was not prevented by either n-3 PUFA supplementation or T_3 administration, substantial decrease in liver injury and oxidative stress was achieved by the combined protocol, which also led to increased liver n-3 PUFA content and decreased n-6/n-3 PUFA ratios, with recovery of NF-κB activity and TNF-α and haptoglobin expression. Prevention of liver IR injury achieved by a combined protocol of T_3 and n-3 PUFA supplementation may represent a novel noninvasive preconditioning strategy with potential clinical application.
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Keywords: n-3 PUFA; Fish oil supplementation; Thyroid hormone; Liver preconditioning; Ischemia–reperfusion injury; Oxidative stress; NF-κB activity

1. Introduction

Oxygen deprivation followed by restoration of oxygen delivery leads to irreversible organ damage [1]. Ischemia–reperfusion (IR) injury is related to diverse clinical conditions, such as myocardial and brain infarct, and major trauma, shock and surgery under vascular occlusion in several organs including the liver [2]. Liver vulnerability, induced in the ischemic period, is associated with severe generation of reactive oxygen and nitrogen species upon oxygen restoration, leading to inflammation underlying the redox activation of nuclear factor-κB (NF-κB) [3]. This latter finding is characterized by (i) an early peak (0.5–3 h of reperfusion) of NF-κB DNA binding activity mainly composed of p50p65 heterodimers triggering inflammatory effects, and (ii) a second peak (9–12 h of reperfusion) composed of p50 homodimers affording cytoprotection by down-regulating the initial p50p65 response, which is lost at later reperfusion times [4]. As liver IR injury occurs in tissue resection, transplantation or trauma, and involves hepatocyte and endothelial cell death, several hepatic preconditioning strategies to reduce liver irreversible damage have been extensively explored, including ischemic preconditioning, pharmacological treatments, gene therapy and mild oxidative stress induction [5-9]. Although surgical maneuvers and pharmacological treatments seemed promising in the experimental setting, their clinical application has been controversial [10,11].

In the last years, we have developed two novel noninvasive preconditioning strategies against IR injury in the rat, involving either the administration of a single dose of L-3,3′,5- triiodothyronine (T_3) or dietary supplementation with n-3 polyunsaturated fatty acids (n-3 PUFA) [12-14], whose derivatives resolvins and protectins have cytoprotective and anti-inflammatory effects [15]. T_3 preconditioning (0.1 mg/kg) occurs 48 h prior to IR (1 h of partial ischemia followed by 20 h of reperfusion) and is triggered by Kupffer cell activation and induction of transient and moderate liver oxidative stress [12,13,16]. n-3 PUFA supplementation, using a protocol of daily doses of 300

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mg/kg during 7 days, also affords significant protection against liver IR injury [14]. Liver protection afforded by both preconditioning strategies, evidenced by normal serum transaminases and liver histologies, was accompanied by the normalization of the IR-altered NF-κB signalling pathway and inflammatory response [12-14]. Taking into account that the preconditioning dose of T_3 and/or the period of n-3 PUFA supplementation might lead to undesirable effects upon clinical application, the present study aimed to evaluate the hepatoprotective effect of n-3 PUFA dietary supplementation plus a lower dose of T_3 against IR injury. For this purpose, we evaluated the preconditioning effect of a combined protocol of fish oil supplementation and thyroid hormone against liver IR injury, in relation to liver NF-κB DNA binding activity and expression of haptoglobin, a NF-κB-controlled antioxidant protein with anti-inflammatory capabilities that is part of the homeostatic acute-phase response of the liver [17].

2. Materials and methods

2.1. Animal treatments and model of partial hepatic IR injury

Male Sprague-Dawley rats (Animal Facility of the ICBM, Faculty of Medicine, University of Chile) weighing 50–70 g were housed in a humidity and temperature-controlled room with 12-h light/dark cycle, allowed free access to water and provided with a specially formulated diet (20% casein, 10% n-6 PUFA, lipo/hydrosoluble vitamins and minerals; Department of Nutrition, Faculty of Medicine, University of Chile). At Day 15, the n-3 PUFA-supplemented groups received daily doses during 3 days of encapsulated fish oil, containing 300 mg n-3 PUFA/kg (General Nutrition, Pittsburgh, PA, USA), and the control group received isovolumetric amounts of saline. At Day 18, the n-3 PUFA-supplemented groups received either a single dose of T_3 (0.05 mg/kg) or isovolumetric amounts of 0.1N NaOH (T_3 vehicle). Under these conditions, the fish oil-supplemented groups received 162.5 mg/kg of eicosapentaenoic acid (C20:5n-3; EPA) and 137.5 mg/kg of docosahexaenoic acid (C22:6n-3; DHA). At Day 20, the animals were anesthetized with Zoletil 50 (tiletamine chlorohydrate 50 mg/kg/zolazepam chlorohydrate 50 mg/kg; Virbac, Carros, France) and subjected to partial liver ischemia by temporary occlusion of the blood supply to the left and median lobes, by means of a Schwartz clip (FST, Vancouver, BC, Canada) for 1 h, followed by 20 h of reperfusion, as previously described [18]. Control animals were subjected to anesthesia and sham laparotomy, thus comprising eight experimental groups: (1) saline-NaOH-sham, (2) saline-NaOH-IR, (3) saline-T_3-sham, (4) saline-T_3-IR, (5) n-3 PUFA-NaOH-sham, (6) n-3 PUFA-NaOH-IR, (7) n-3 PUFA-T_3-sham and (8) n-3 PUFA-T_3-IR. At the end of the reperfusion period, blood samples were obtained by cardiac puncture, for serum aspartate amino transferase (AST) and alanine amino transferase (ALT) assessments. Liver samples were taken from the medial lobes, frozen in liquid nitrogen and stored at −80°C for EMSA, Western blot and PCR assays (NF-κB and haptoglobin), or fixed in phosphate-buffered formalin, embedded in paraffin and stained with hematoxylin–eosin (morphology assessment). Experimental animal protocols and animal procedures complied with the Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, NIH Publication 86-23, revised 1985) and approved by the Bioethical Committee (Faculty of Medicine, University of Chile, CBN No. 0274 FMUCH).

2.2. Assessment of liver injury

Serum AST and ALT were measured using specific commercial kits (Valtek Diagnostics, Santiago, Chile) and expressed as unit per liter. Liver morphological assessment was carried out in liver samples sliced from paraffin blocks, stained with hematoxylin–eosin and evaluated by a pathologist (double blind assay) in order to determine hepatocellular necrosis and the score for liver injury.

2.3. Fatty acid analysis

For fatty acid analysis, the liver samples were homogenized in distilled water and the lipid components were extracted with a 1:2 chloroform/methanol solution, followed by centrifugation (2000×*g*, 10 min, room temperature). After extraction of the chloroformic phase, the solvent was allowed to evaporate and the samples were stored at −20°C [19]. Prior to the gas–liquid chromatography assay, fatty acids from liver phospholipids were methylated by incubation (100°C) with BF_3 methanol (14%) and the fatty acid methyl esters (FAME) were extracted with hexane. After evaporation with nitrogen and resuspension in dichloromethane, the samples were stored at −20°C until the gas–liquid chromatography assay [20]. A Hewlett-Packard gas chromatograph (model 7890 A series II plus), equipped with a capillary column (J and W DB-FFAP, 30 m×0.25 mm; I.D. 0.25 μm), automatic injector and flame ionization detector, was used for FAME separation and detection. Identification of FAME was carried out by comparison of their retention times with those of individual purified standards and values were expressed as grams per 100 grams of FAME.

2.4. Liver parameters related to oxidative stress

In anesthetized animals, livers were perfused *in situ* with a cold solution containing 150 mM KCl and 5 mM Tris (pH 7.4) to remove blood, and total reduced glutathione (GSH) [21], protein carbonyl and total protein contents were measured [22].

2.5. NF-κB electromobility shift assay

Nuclear protein extracts from liver samples were prepared according to Deryckere and Gannon [23]. The samples were subjected to electromobility shift assay for assessment of NF-κB DNA binding using the NF-κB probe 5′-GAT CTC AGA GGG GAC-TTT CCG AG-3 (Invitrogen Life Technologies, Carlsbad, CA, USA), labeled with α-32PdCTP using Klenow DNA Polymerase Fragment I (Invitrogen), as described previously [24]. The specificity of the reaction was determined by a competition assay using 100-fold molar excess of unlabeled DNA probes. Samples were loaded on nondenaturating 6% polyacrylamide gels and run until the free probe reached the end of the gel. NF-κB bands were detected by autoradiography and quantified by densitometry using Scion Image (Scion, Frederick, MD, USA).

2.6. Western blot analysis of haptoglobin

Liver samples (100–500 mg) frozen in liquid nitrogen were homogenized and suspended in a buffer solution (pH 7.9) containing 10 mM HEPES, 1 mM EDTA, 0.6% Nonidet P-40, 150 mM NaCl and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, 1 μg/mL leupeptin and 1 mM orthovanadate). Soluble protein fractions (10 μg) were separated on 12% polyacrylamide gels using SDS-PAGE and transferred to nitrocellulose membranes [25], which were blocked for 1 h at room temperature with TBS containing 5% nonfat dry milk. The blots were washed with TBS containing 0.1% Tween 20 and hybridized with rabbit polyclonal antibodies for human haptoglobin (Dako, Carpinteria, CA, USA). In all determinations, mouse monoclonal antibody for rat β-actin (ICN Biomedicals, Aurora, OH, USA) was used as internal control. After extensive washing, the antigen–antibody complexes were detected using horseradish peroxidase-labeled goat anti-rabbit IgG or goat antimouse IgG and a SuperSignal West Pico Chemiluminescence kit detection system (Pierce, Rockford, IL, USA).

2.7. Reverse transcription–polymerase chain reaction (RT-PCR) assay for haptoglobin and tumor necrosis factor-α (TNF-α)

Total liver RNA was extracted with RNAqueous-4PCR Kit (Ambion, Austin, TX, USA) and quantified by measurement of ultraviolet absorption at 260 nm. For RT-PCR assay of mRNA, first-strand cDNA was synthesized from total RNA (0.5 μg) using Thermoscript-Reverse Transcriptase (Invitrogen) and random hexamer primers (Promega, Madison, WI, USA). cDNA was amplified in a PCR reaction using Taq Platinum (Invitrogen) in the presence of primers specific for rat haptoglobin. Nucleotide sequences for sense and antisense primers used were 5′-CTA CAG ACC GAA GGA GAT GG-3′ and 5′-GGC AGA TAG GCA TGA CTT TC-3′ for haptoglobin and 5′-ACT GAA CTT CGG GGT GAT CG-3′ and 5′-TAC ATG GGC TCA TAC CAG GG-3′ for TNF-α (Invitrogen), respectively. In these conditions, a 451-bp (base pairs) for rat haptoglobin and a 441-bp for TNF-α cDNA were amplified. Serum levels of TNF-α were measured by ELISA (Ultrasensitive Cytoscreen KRC 3013 kit, Biosource). To control the relative amount of total mRNA transcribed in each reverse transcriptase reaction, an RNA 18S invariant standard [Classic II 18S Internal Standards (324 bp); Ambion] was used. PCR conditions included denaturation, annealing and extension at 94°C, 56°C and 72°C, for 30, 30 and 60 s, respectively, for 38 cycles. PCR products were electrophoresed on 2% agarose gels containing ethidium bromide, visualized by UV-induced fluorescence and analyzed by densitometry using Scion Image (Scion) [26]. Band analysis was performed also with Scion Image (Scion).

2.8. Statistical analysis

Statistical analysis was performed with GraphPad Prism 4.0 software (GraphPad Software, San Diego, CA, USA). Values shown represent the mean±S.E.M. for the number of separate experiments indicated. One-way ANOVA and the Newman–Keuls test assessed the statistical significance of differences between mean values. A *P* value of less than .05 was considered significant.

3. Results

3.1. T_3 modifies thyroid hormone serum levels, calorigenesis and liver oxidative stress status

Rats subjected to a single dose of T_3 (0.05 mg/kg) achieved a significant elevation in serum T_3 levels within 24 h, returning to control values at 36 h (Fig. 1A). Under these conditions, T_3 administration led to a calorigenic response evidenced by significant increases in the rectal temperature of the animals that lasted for

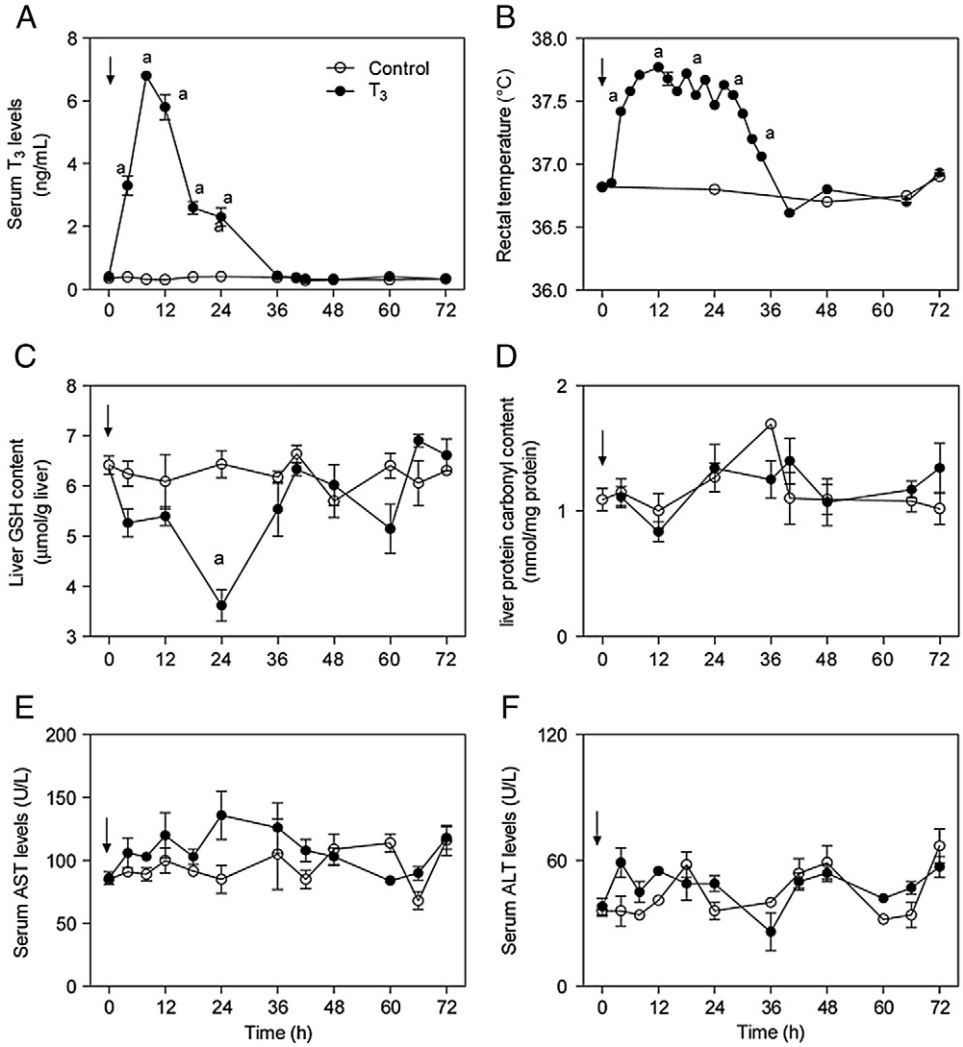

Fig. 1. Time-course study of the effects of T_3 administration on (A) serum T_3 levels. (B) Rectal temperature. (C) Liver GSH content. (D) Liver protein carbonyl content, and serum (E) AST and (F) ALT levels in the rat. T_3 (0.05 mg/kg intraperitoneally) or T_3 vehicle (controls) was given at Time 0 (arrows). Data are expressed as means±S.E.M. for 3 to 14 animals per group. [a]$P<.05$ compared with the respective time controls or with average control data at Time 0.

34 h, leveling off after 36 h (Fig. 1B). In concomitance, within 24 h after T_3 administration, oxidative stress in the liver was triggered, as shown by significant GSH depletion at 24 h (Fig. 1C). All these changes occurred without major alteration in protein carbonylation (Fig. 1D) and in the serum levels of AST (Fig. 1E) and ALT (Fig. 1F).

3.2. n-3 PUFA supplementation modifies liver fatty acid pattern

Control animals subjected to n-3 PUFA have a significant increment in the total content of PUFAs compared with the other experimental groups (Table 1). Liver fatty acid pattern in control rats

Table 1
Fatty acid composition of liver total lipids in control rats and animals subjected to EPA plus DHA supplementation under the influence of T_3 and ischemia–reperfusion (IR)

	Groups							
	Saline/NaOH/sham (a)	Saline/NaOH/IR (b)	Saline/T_3/sham (c)	Saline/T_3/IR (d)	n-3 PUFA /NaOH/sham (e)	n-3 PUFA /NaOH/IR (f)	n-3PUFA /T_3/sham (g)	n-3PUFA /T_3/IR (h)
SAFA	1.87±0.16	2.26±0.22	1.64±0.013	2.12±0.006	2.37±0.25	2.06±0.15	2.06±0.28	1.50±0.14
MUFA	0.64±0.22	1.12±0.36	0.35±±0,12	0.64±0.23	1.02±0.02	0.62±0.001	0.51±0.05	0.42±0.03
PUFA	3.23±0.17(d,e,h)	2.72±0.4	2.2±0,04	1.94±0.05	4.92±0.04 (a–d,f–h)	3.04±0.14	2.74±0.37	1.97±0.18
n-6 PUFA	2.63±0.03	3.3±0.46 (d,h)	2.01±0.04	1.41±0.08	4.13±0.07 (c–h)	2.11±0.03	2.24±0.32	1.64±0.13
n-3 PUFA	0.33±0.04 (d)	0.35±0.05 (d)	0.31±0.003 (d)	0.12±0.001	1.04±0.04 (a–d,f–h)	0.63±0.04 (b–d,h)	0.50±0.05 (d)	0.33±0.05 (d)
EPA+DHA	0.28±0.03 (d)	0.30±0.01 (d)	0.3±0.002 (d)	0.1±0.001	0.98±0.03 (a–d,f–h)	0.6±0.03 (a–d,g,h)	0.45±0.04 (b–d,h)	0.3±0.03 (d)
n-6/n-3 PUFA ratio	8.194±0.9 (e–h)	8.23±0.63 (e–h)	6.58±0.21	11.63±0.72 (a–c,e–h)	3.99±0.09	3.38±0.29	4.42±0.34	5.12±0.40

n-3 Supplementation and/or T_3 treatment effect over liver lipid profile. Values, expressed as g/100 g fatty acid methyl esters, represent means±S.E.M. for four to seven animals per group. Saturated fatty acids (SAFA) are 12:0, 16:0, 18:0, 20:0, 22:0 and 24:0. Mono-unsaturated fatty acids (MUFA) are 14:1,n-7, 16:1,n-7, 18:1,n-9, 20:1,n-9, 22:1,n-9 and 22:1,n-9. Polyunsaturated fatty acids (PUFA) are 18:2,n-6, 18:3,n-6, 18:3,n-3, 20:2,n-6, 20:3,n-6, 20:3,n-3, 20:4,n-6, 20:5,n-3 (eicosapentaenoic acid, EPA) and 22:5, n-3 (docosahexaenoic acid, DHA). Significance ($P<.05$) is shown by the letters (a-h) identifying each group.

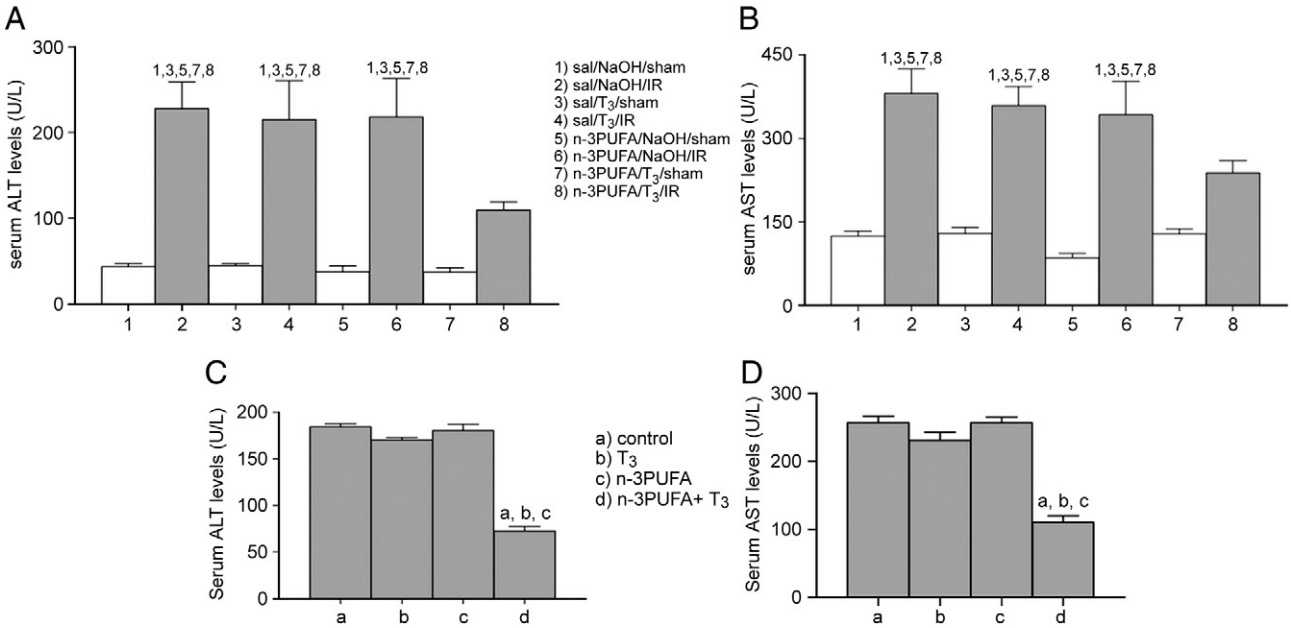

Fig. 2. Effects of T_3 administration plus EPA+DHA supplementation on serum (A) ALT and (B) AST levels after 1 h of liver ischemia and 20 h of reperfusion. Values are shown as means±S.E.M. for 9 to 13 animals per group. Net changes induced by IR in ALT (C) and AST (D) after IR in nonsupplemented rats and animals subjected to T_3 plus EPA+DHA were calculated as described in Materials and Methods. Significance ($P<.05$) is shown by the numbers identifying each group.

subjected to IR was similar to that of control sham-operated animals (Table 1). Levels of hepatic n-6 PUFA exhibited a significant diminution in animals subjected to T_3 administration and/or IR (Table 1). n-3 PUFA supplementation enhanced the hepatic content of EPA plus DHA by 3.5-fold ($P<.05$), with significant diminution in the n-6/n-3 PUFA ratio ($P<.05$) over control values, in all n-3 PUFA-supplemented animals (Table 1). None of these parameters was further modified by IR (Table 1).

3.3. T_3 administration and n-3 PUFA supplementation suppress liver injury induced by IR

3.3.1. Parameters of liver injury

In relation to control sham-operated animals, IR in control, T_3-treated and n-3 PUFA-supplemented animals led to extensive liver injury, as shown by a 5.2-, 4.8- and 5.8-fold increase in serum ALT, respectively (Fig. 2A), and by a 3.1-, 2.8- and 4.0-fold increase ($P<.05$)

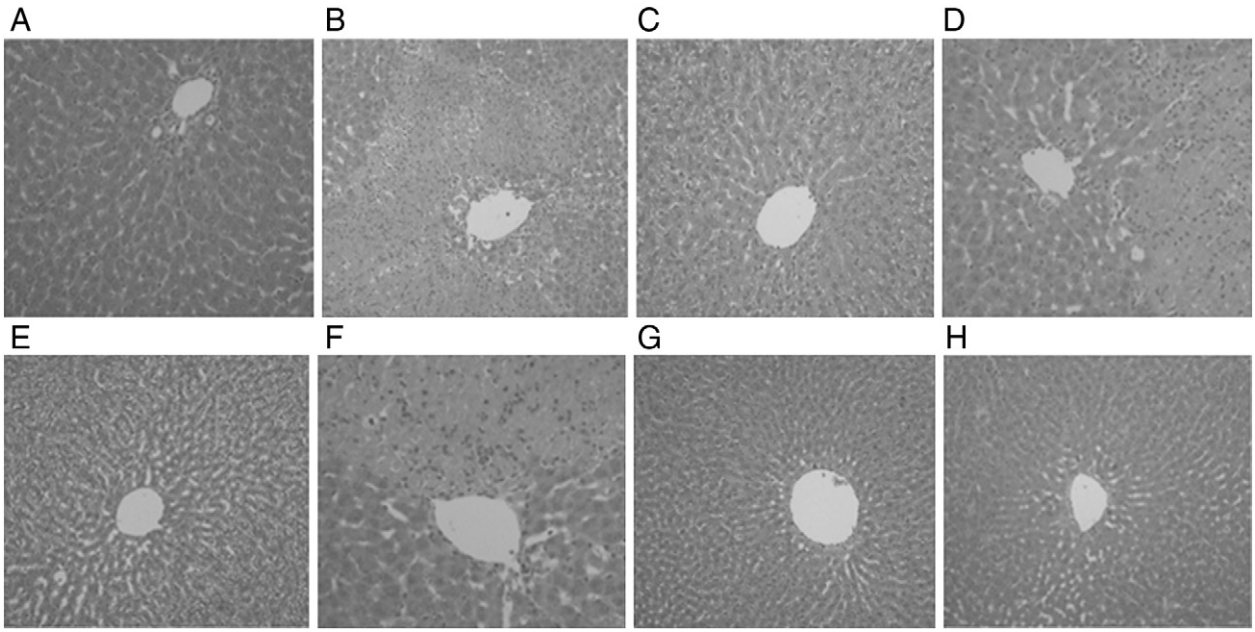

Fig. 3. Effect of the combined protocol T_3+n-3PUFA on liver histology after hepatic ischemia–reperfusion in treated animals and controls. Representative liver sections from (A) control sham, (B) control IR, (C) saline-T_3-sham, (D) saline-T_3-IR, (E) n-3PUFA-NaOH-sham, (F) n-3PUFA-NaOH-IR, (G) n-3PUFA-T_3-sham and (H) n-3PUFA-T_3-IR. (Hematoxylin–eosin-stained liver sections from a total of four animals per experimental groups were analyzed, original magnification ×20.)

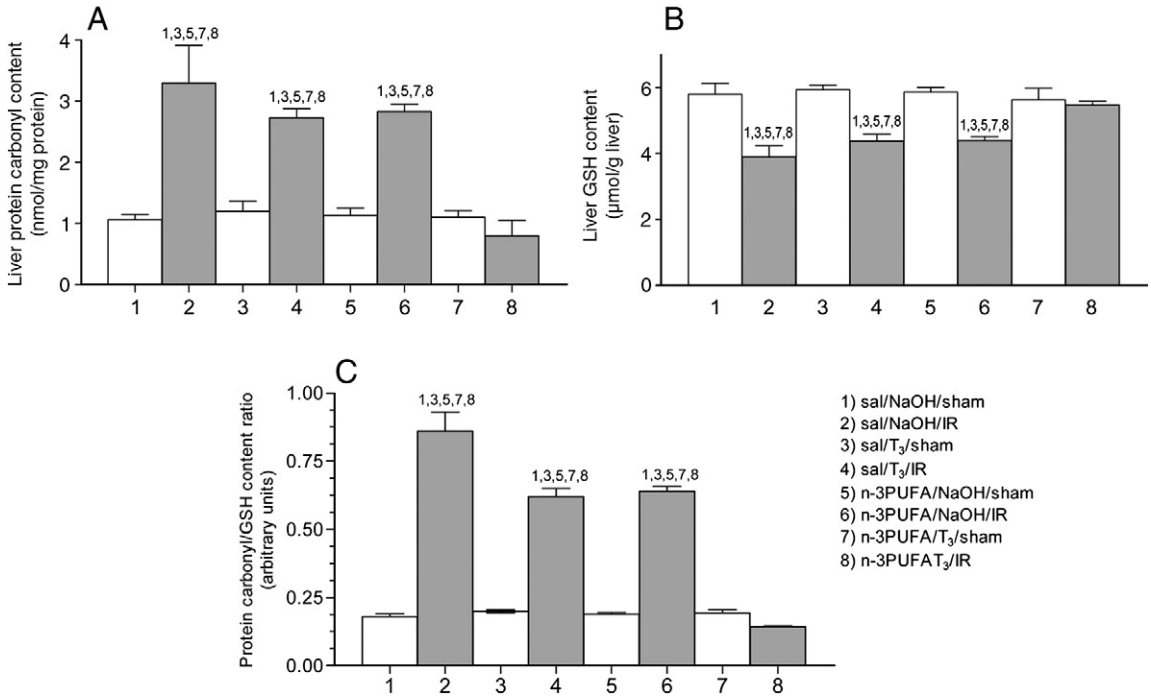

Fig. 4. Effect of n-3PUFA supplementation and/or T_3 treatment on (A) liver protein carbonyl content, (B) total GSH content and (C) the respective protein carbonyl/GSH content ratio after IR in treated and nontreated animals. Values are expressed as means±S.E.M. for 3 to 14 animals per group. Significance ($P<.05$) is shown by the numbers identifying each group.

in serum AST, respectively (Fig. 2B). n-3 PUFA supplementation followed by T_3 administration led to AST and ALT values comparable to those in control animals (Fig. 2A and B, respectively), thus eliciting significant net diminutions in relation to control-IR, T_3-IR and n-3 PUFA-IR animals (Fig. 2C and D). In agreement, histological assessment of the liver from the studied groups showed that control-sham (Fig. 3A), T_3-sham (Fig. 3C), n-3 PUFA-sham (Fig. 3E) and n-3 PUFA-T_3-sham (Fig. 3G) animals exhibited normal liver morphology, whereas IR in these animals resulted in substantial distortion of liver architecture, with extensive perivenular necrosis

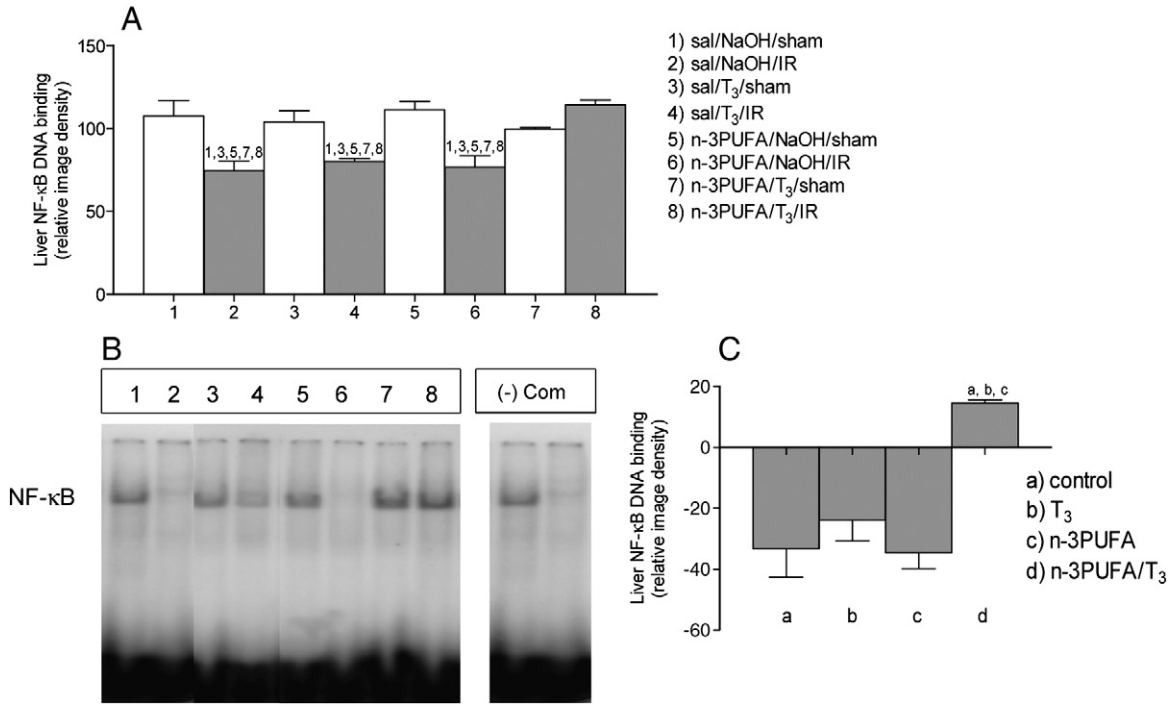

Fig. 5. Effect of n-3PUFA supplementation and/or T_3 treatment on liver NF-κB DNA binding activity on electromobility shift assay. (A) Bar graphs corresponding to means±S.E.M. of densitometric quantification of relative DNA binding for four animals in each group. (B) Autoradiographs representing lanes loaded with 8 μg of nuclear protein from a representative animal in each experimental group in competition experiments without (−) and with (com) 100-fold molar excess of the unlabeled DNA probe. (C) Net changes induced by the different treatments. Significance ($P<.05$) is shown by the numbers (A) or letters (C) identifying each group.

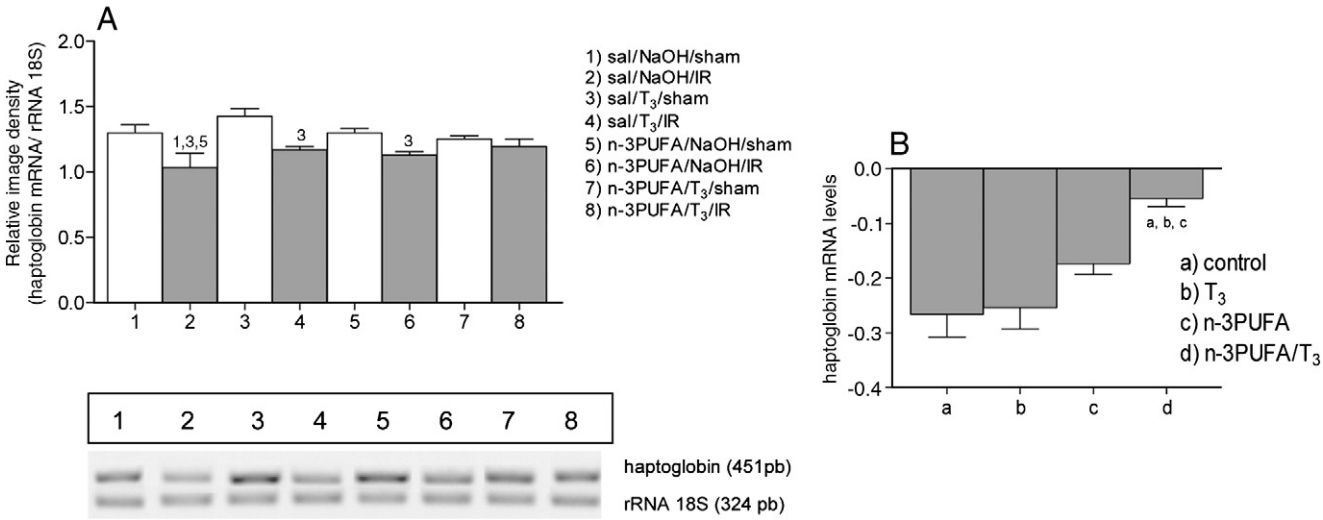

Fig. 6. Effect of n-3PUFA supplementation and/or T_3 treatment on liver haptoglobin RNA expression after hepatic IR injury in treated and nontreated animals. (A) Bar graphs, corresponding to means±S.E.M. for four animals in each group, of densitometric quantification of RT-PCR products of haptoglobin mRNA, expressed as haptoglobin mRNA/18S rRNA ratios to compare lane–lane equivalency in total RNA content (B). Representative agarose gel electrophoresis of the RT-PCR products for haptoglobin mRNA (451 bp) and for 18S rRNA (324 bp) after ethidium bromide staining in total liver RNA samples. (C) Net changes induced by the different treatments. Significance ($P<.05$) is shown by the numbers (A) or letters (C) identifying each group.

areas and inflammatory infiltrate (Fig. 3B, D and F). On the contrary, normal architecture, no necrotic areas with isolated and disperse inflammatory infiltrates were observed in the livers of n-3 PUFA-T_3-IR animals, thus representing a decreased injury score (Fig. 3H).

3.4. T_3 administration and n-3 PUFA supplementation normalize liver oxidative stress, NF-κB DNA binding activity, haptoglobin expression, and serum TNF-α levels

In relation to control sham-operated animals, IR in control, T_3-treated and n-3 PUFA-supplemented animals led to significant diminution in liver oxidative stress (Fig. 4), NF-κB DNA binding activity (30.9%, 23.0% and 31.0%, respectively) (Fig. 5A) with net decreases of 33.3±9.3 (n=7), 24.0±6.7 (n=4) and 34.7±5.1 (n=8) arbitrary units, respectively (Fig. 5C). Competitive analysis confirmed the specificity of the assay (Fig. 5B). IR in n-3 PUFA-T_3-preconditioned rats resulted in normalization of this parameter as evidenced by values of the NF-κB DNA binding activity comparable to those in control animals (Fig. 5A). Furthermore, a significant ($P<.05$) net enhancement of 14.6±1.0 (n=4) arbitrary units (Fig. 5C) was found. Consistent with these results, significant diminution in liver haptoglobin expression (RT-PCR analysis) was found after IR, when values of control, T_3-treated and n-3 PUFA-supplemented animals are compared to control sham-operated groups, thus leading to 20.5%, 17.8% and 13.3% decreases, respectively (Fig. 6A), and net decreases of 0.27±0.04 (n=10), 0.25±0.04 (n=9) and 0.17±0.02 (n=9) arbitrary units, respectively (Fig. 6B). In agreement, the hepatic content (Western blot analysis) of haptoglobin was also significantly decreased in these experimental groups (10.6%, 10.4% and 7.5%, respectively) (Fig. 7A) with concomitant net decreases of 0.13±0.03 (n=7), 0.11±0.02

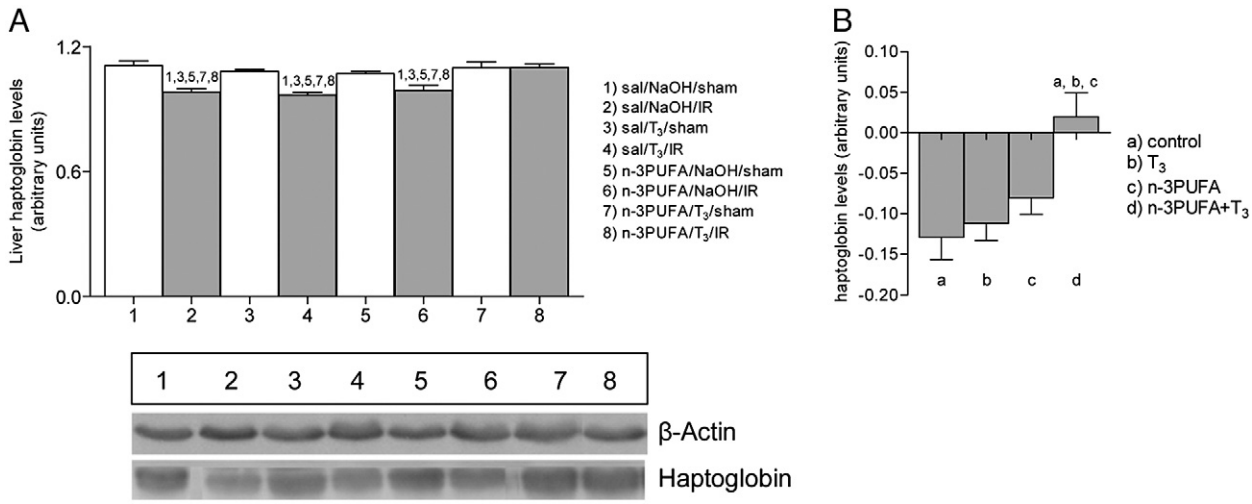

Fig. 7. Effect of n-3PUFA supplementation and/or T_3 treatment on liver haptoglobin expression evaluated via Western blotting after hepatic IR injury in treated and nontreated animals. (A) Bar graphs, corresponding to means±S.E.M. for four to six animals in each group, of densitometric analysis of bands, expressed as haptoglobin/β-actin ratios to compare lane–lane equivalency in total protein content. (B) Representative blots of haptoglobin/β-actin are shown, using 25 μg of soluble protein from different animals in each group studied. (C) Net changes induced by the different treatments. Significance ($P<.05$) is shown by the numbers (A) or letters (C) identifying each group.

(n=3) and 0.08±0.02 (n=3) arbitrary units, respectively, triggered by IR (Fig. 7B). Interestingly, n-3 PUFA-T_3 preconditioning normalized both haptoglobin expression (Fig. 6A) and the liver content of the protein (Fig. 7A), as evidenced by values comparable to those found in control animals (Figs. 6A and 7A). n-3 PUFA-T_3 preconditioning decreased the net effects of IR in the expression of haptoglobin [0.054±0.01 (n=8); P<.05] (Fig. 6B). In relation to the net effects of IR in the content of the protein, n-3 PUFA-T_3 preconditioning determined a significant net enhancement of 0.02±0.03 (n=4) arbitrary units in this parameter (Fig. 7B). IR increased (P<.05; n=6, experimental group) serum TNF-α levels [saline-NaOH-sham, 21.6 ±3.7 pg/ml; saline-NaOH-IR, 39.9±4.6; n-3 PUFA-T_3-sham, 23.2±3.7; and n-3 PUFA-T_3-IR, 20.7±4.1] and liver TNF-α mRNA levels [saline-NaOH-sham, 2.17±0.14 arbitrary units; saline-NaOH-IR, 4.47±0.31; n-3 PUFA-T_3-sham, 2.05±0.05; and n-3 PUFA-T_3-IR, 3.00±0.26], which was abolished by T_3 and n-3 PUFA preconditioning.

4. Discussion

In agreement with previous studies, the liver IR protocol used in this study induced major changes in parameters related to liver injury, oxidative stress, NF-κB signaling and TNF-α response [12-14,16]. Liver IR injury has been related to reduction in NF-κB activation and loss of the cytoprotective acute-phase response or lack of changes in inducible nitric oxide synthase expression and activity [12,26].

We had previously demonstrated significant protection against liver IR injury after a single dose of 0.1 mg/kg of T_3 or dietary supplementation with n-3 PUFA for 7 days [12-14]. Although lack of protection against liver IR injury was observed after a single dose of 0.05 mg/kg T_3 or n-3 PUFA supplementation for 3 days, significant protection was achieved by a combined protocol of both, as shown by normal liver histologies, oxidative stress status and serum transaminase activities. To study the mechanisms of protection involved in liver preconditioning triggered by the combined actions of n-3 PUFA and T_3, we assessed NF-κB DNA binding capacity and haptoglobin expression. These studies showed that decreased liver damage after n-3 PUFA supplementation and T_3 administration was accompanied by normalization of NF-κB signaling and TNF-α response, with values of NF-κB DNA binding activity, haptoglobin mRNA expression and content, and serum TNF-α levels and liver TNF-α mRNA content being similar to those of control sham-operated animals. Recent reports on the effects of long-term supplementation of dietary fish oil suggest enhancement of thyroid hormone action by fish oil supplementation, as shown by higher expression of liver thyroid hormone receptor β1 protein and enhanced activity of liver mitochondrial glycerophosphate dehydrogenase, the enzyme involved in thermogenesis and a well-characterized target of thyroid hormone, stimulated by T_3 via thyroid hormone receptor β1 [27]. Although the effects of short-term n-3 PUFA supplementation on thyroid signaling via thyroid hormone receptor β1 have not been assessed, the molecular mechanisms responsible for liver preconditioning after n-3 PUFA supplementation and T_3 administration may include enhancement of thyroid hormone action by fish oil supplementation.

Emerging evidence also suggests that thyroid hormone could improve postischemic cardiac function [28]; however, the experimental and clinical evidence has been contradictory to support the use in humans [29], a fact that could be related to the dose-dependent adverse effects of the hormone over the heart rate and metabolism. n-3 PUFAs are dietary components with important roles in several physiological processes, probably due to the n-3 PUFA derivatives resolvins and protectins, which have cytoprotective properties that may be important in the preservation of cellular integrity related to IR-induced liver injury [15]. Both n-6 and n-3 PUFAs are cellular lipids and substrates for the synthesis of physiological mediators. α-Linolenic acid (C18:3n-3) is the predominant dietary n-3 PUFA and the precursor for the longer-chain n-3 PUFAs eicosapentaenoic acid (C20:5n-3; EPA) and docosahexaenoic acid (C22:6n-3; DHA). Both EPA and DHA have anti-inflammatory and tissue protective effects [15], which may involve a direct antioxidant action due to their high susceptibility to free-radical reactions [30]. This view is supported by the decreasing effect of IR on the hepatic levels of n-3 PUFA in n-3 PUFA-supplemented animals, both in the control group (38% diminution) and in T_3-treated rats (33% decrease) (Table 1).

Collectively, data reported here support a role for short-term n-3 PUFA supplementation followed by a low dose of T_3 in preventing liver injury induced by IR, a noninvasive preconditioning strategy with clinical potential, as adverse cardiovascular effects triggered by T_3 may be decreased by combining T_3 and n-3 PUFA hepatoprotective effects. The synergistic effect of combined n-3 PUFA and T_3 administration may be due to the attainment of an oxidative stress and NF-κB activation status suitable for up-regulating cytoprotective functions and limiting inflammatory responses, thus enhancing the resistance of the liver against IR injury. The underlying mechanisms may involve an n-3 PUFA-mediated enhancement of both the anti-inflammatory potential of the liver, possibly through resolvin production [15], and its antioxidant status [31], with concomitant up-regulation of the expression of acute-phase proteins (haptoglobin) [32] and antioxidant or anti-apoptotic components by T_3 [33], which may abrogate the damaging effects of the early phase of IR.

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